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A NEW METHOD OF SILVER IMPREGNATION FOR NERVE CELLS AND FIBERS

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ABSTRACT.—**Fragments of tissue, immediately after death, are fixed in Debaisieux's modification of the Duboscq-Brazil picro-acetic-formol fluid, and treated as follows: Hydrate by soaking 2–6 hr. in distilled water with 30 drops of conc. NH_4OH per 100 cc. Freeze and cut sections about 25 μ in thickness. Bleach sections about 15 min. in ammoniacal water (52 drops conc. NH_4OH per 100 cc. water). Transfer to 20% $AgNO_3$ solution and heat at 45° C. till light brown. Add conc. NH_4OH drop by drop till the Ag precipitates and then redisolves into an opalescent solution. Pour solution and sections into a little distilled water and transfer sections quickly to formaldehyde solution (3 cc. formalin to 100 cc. water). Dip sections in distilled water and transfer to 1% aqueous gold chloride till deep blue. Place for about 10 minutes in 5% aqueous sodium thiosulfate solution for fixing and clearing. Wash thoroly in tap water, dehydrate and mount. Special directions are given for applying this technic to delicate material such as insects, and for use with serial sections.**

This method and its application on free sections has recently been published.[1] An application of this method to sections on slides will be given hereafter.

The method is based on the same principle as Dr. Dewulf's recently published discovery,[2] i.e. the use of a nascent silver complex instead of the stable combination. We have preferred, however, in the place of Hortega's sodium carbonate, to use pure ammonia for the precipitation of the silver nitrate as in Rogers' method, the impregnations thus obtained being much more energic and complete, especially where invertebrate material is concerned.

[1]Debauche, H. R. 1939. Nouvelle méthode d'imprégnation des cellules nerveuses par le nitrate d'argent. Ann. Soc. Sci. Bruxelles. Ser. II, 59, 23–27.

[2]Dewulf, A. 1938. Une nouvelle méthode d'imprégnation des neurofibrilles. Journ. Belg. Neurol. Psychiâtric. 1938, No. 6, 407–410.

The age of the animal is unimportant. The fragments of tissue to be impregnated must be fixed immediately after death. The best results are obtained with material fixed in Debaisieux's modification of the Duboscq-Brazil[3] fluid, i.e.:

Ethyl alcohol 80%	100 cc.
Picric Acid	2 g.
Acetic Acid	10 cc.
Formaldehyde	40 cc.

Good results have also been obtained on material fixed in formaldehyde (Merck's pro Analysi) 10 to 20%.

It is advisable to use only the purest obtainable chemicals; Merck's "Pro Analysi"[4] products have given satisfying results.

The material must be preserved in the fixing fluid as long as convenient. A long fixation will give the best results. It has been demonstrated that, in material fixed for a short time only, the background of the preparation shows a tendency to take on the impregnation, obscuring more or less the finest details. This tendency disappears gradually by lengthening the period of fixation. A fixation of 100 days will give very clear preparations; splendid results were obtained with material fixed and kept in Duboscq-Brazil fluid for over one year.

Before sectioning, the pieces are soaked in distilled water with 30 drops of conc. ammonia per 100 cc. This hydrating process must last from two to six hours according to size of pieces.

While this hydration is in progress, prepare the following solutions:

1. *Ammoniacal water:* Distilled water ..... 100 cc.
   Ammonia (Merck's pro analysi) .. 52 drops.
2. *Silver nitrate:* Distilled water ..... 20 cc.
   Silver nitrate (Merck's pro analysi) .... 4 g.
3. *Formaldehyde solution for reduction:*
   Formaldehyde (Merck's pro analysi) .. 3 cc.
   Distilled water ..... 100 cc.
4. *Yellow gold chloride:* 1% aqueous.
5. *Sodium hyposulfide* (sodium thiosulfate): 5% aqueous.

When the pieces are sufficiently hydrated, proceed as indicated below:

a) Section by freezing technic (25 μ has proved to be the most convenient thickness).

[3]Debaisieux, P. 1935. Organes scolopidiaux des pattes d'insectes. I. Lépidoptères et Trichoptères. La Cellule, 44, 273–314.

[4]This correspondes to the "reagent" grade of the American company of the same name.

b) The sections are received in a dish containing the ammoniacal water. They are left to bleach in this bath for about 15 minutes.
c) Transfer the sections to the silver nitrate solution, in an Erlenmeyer flask.
d) Continually agitating, heat moderately (45° C.), till the sections become light brown.
e) Always agitating, precipitate the silver nitrate slowly in the solution containing the sections, by addition of pure ammonia one drop at a time. Then redissolve the precipitate carefully by adding more ammonia. This process must be stopped when the stage of opalescence is attained.
f) When this stage is reached, pour the solution with the sections immediately into a dish containing a little distilled water.
g) Transfer the sections rapidly to the formaldehyde solution, where the reduction takes place. A good reduction is recognized by the uniform, rich brown color which the sections take in this solution.
h) The sections are then dipped in distilled water and transferred immediately to the gold chloride solution. They are left in this bath till their color changes to a deep blue.
i) Transfer the sections to the sodium thiosulfate solution where they are fixed and cleared, about 10 minutes. Then wash abundantly in tap water.
j) Dehydrate and mount in neutral Canada balsam, with cover glass.

If the impregnation has been correctly performed, the nerve cells and intraprotoplasmatic neurofibrillae appear dark blue on a pink, almost colorless ground. The nuclei are transparent, well defined.

For delicate material, such as insects, of which frozen sections are very difficult to obtain, it is generally better to proceed as follows:

a) Place pieces 24 hours in: Alcohol 90% . . . . . 50 cc.
Conc. ammonia . . . . . 15 drops.
b) One hour and a half in absolute alcohol.
c) 20 minutes in toluol.
d) Six hours in paraffin with 20% beeswax, in the oven (58° C.).
e) Imbed in same paraffin.
f) The sections are cut as usual with paraffin imbedded material, but the ribbon is transferred directly in xylol which dissolves the paraffin, thus freeing the sections.
g) The sections are transferred into absolute alcohol, to which distilled water is added, very carefully and slowly, one drop at a time. Water must be thus added until all alcohol has been removed and

the sections are thoroly hydrated. The impregnation can then be carried on as on frozen sections.

Application of the Method to Serial Sections on Slides

The material must be fixed in Debaisieux's modification of the Duboscq-Brazil fluid. The pieces are imbedded in paraffin as indicated above. The sections must be carefully spread on the slide, to ensure sufficient adherence.

a) The paraffin is removed by xylol and the sections are hydrated, using only chemically pure alcohol.
b) The slide is brought into ammoniacal water (distilled water 50 cc.; conc. ammonia 26 drops), and left in this bath for about 10 minutes.
c) Dip slide in distilled water and transfer into a 10% solution of silver nitrate. This bath is heated to 45° C. The slide is left in this solution about 10 minutes, till the sections become light brown.
d) Transfer the slide immediately to a silver ammoniacal complex prepared in advance. This solution is prepared as in Roger's method, but using only a 10% solution of silver nitrate. The slide is left in this bath from 1–5 minutes.
e) Dip slide in distilled water and transfer immediately to reducing bath, i.e.: 3% solution of pure formaldehyde.
f) Tone in gold chloride (1%).
g) Fix in 5% solution of sodium thiosulfate.
h) Wash well in tap water.
i) Dehydrate and mount in neutral Canada balsam, with cover glass.

This method has been successfully tried on man, rabbit, rat, different birds and numerous invertebrates, especially insects.

A QUADRUPLE STAIN COMBINATION FOR PLANT TISSUES

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ABSTRACT.—**A new procedure for the quadruple staining of paraffin sections of plant tissues is offered, involving the use of safranin, methyl violet, fast green and orange G. Differentiation is practically automatic and is controlled by the use of special washing solutions. Striking color-contrast effects permit the identification of every type of cell structure and cytoplasmic inclusions.**

Quadruple stain combinations are still comparatively new in botanical microtechnic. One was devised by Dr. Geo. H. Conant (outlined in *Triarch Topics* for March, 1938), but the writer found that the excessive use of clove oil solutions in this procedure was objectionable for the reason that it is very difficult to remove absolutely every trace of the clove oil, with the result that fading sets in sooner or later. An effort was therefore made to devise a different procedure using the same dyes, and also to minimize as far as possible the personal judgment factor.

The dyes used in quadruple staining include safranin O, fast green FCF, methyl violet 2B and orange G. A fifth stain can, if desired, be added in the form of Harris' hematoxylin; this is used before the other stains and should not be differentiated as far as usual since it will become somewhat weakened during the differentiation of the other dyes. Crystal violet may be substituted for the methyl violet, and gold orange for the orange G.

The disadvantage inherent in any quadruple staining procedure is that washing solutions become saturated with excess stain after only a few slides have been passed thru them. Water alone cannot be used for all the washes; otherwise those stains already applied would be removed or their intensity considerably lessened. The necessity for frequently replacing washing solutions would make any procedure unduly expensive; consequently it is desirable to employ relatively inexpensive reagents for removing excess stain solutions.[1] Given an inexpensive washing reagent, it must then be one which does not have any effect upon the affinity of any dye for the structures which

[1]Of the solvents used in the present work, the methyl cellosolve was a Carbide and Carbon Chemicals Corp. product and the tertiary butyl alcohol was obtained from the California Botanical Materials Co. The prices are respectively $2.40 and $2.10 per gallon; the cheaper USP clove oil is entirely satisfactory, and with 95% ethyl alcohol costing about 56 cents per gallon, the washes are not unduly expensive.

it is supposed to stain, but it should remove the dye from all other structures for which it normally has no affinity and leave them clear for staining by other dyes. On the other hand, the staining solutions must have their staining capacities enhanced by the addition of other reagents; in other words, any dye which is more of a general than a selective stain must have its affinity accentuated. Overlapping or mixing of stain affinities is very undesirable. When used on plant tissues, safranin, for example, tends to be more of a general than a selective stain and therefore requires the addition of other substances to cause it to become more readily differentiated.

Only tissues in which there is a variety of cell types or considerable chemical difference in the structure of the cells and the cytoplasmic inclusions are adaptable for quadruple staining. Relatively undifferentiated tissues merely show the general effect given by a Flemming triple combination. If no starch is present, for instance, there is little reason for using the violet dye, but it must be mentioned that starch grains and plastids have been found in totally unexpected places as a result of applying quadruple combinations.

The procedure, as finally revised after trial on a wide variety of plant tissues, is as follows. The solutions are somewhat complicated, but the actual procedure is simple.

1. Bring slides bearing paraffin sections down to 70% ethyl alcohol.

2. Stain in safranin O for 24–48 hours. Overstaining is rarely possible except with certain Gymnosperm structures. With the latter, the time should be reduced to 2 hours. To make the safranin solution,[2] dissolve 4 g. certified safranin O dye (the writer used a National Aniline and Chemical Co. sample, No. NS-10) in 200 cc. methyl cellosolve (ethylene glycol monomethyl ether), then add 200 cc. 50% ethyl alcohol, 15 cc. formalin and 3 g. sodium acetate. The formalin acts as a mordant, the sodium acetate accentuates the brilliancy of the dye, and together they permit an unusually sharp differentiation.

3. Rinse in tap water. This step should not be omitted, otherwise the dye may become irremovably precipitated.

4. Stain in 1% methyl violet 2B (a National Aniline sample, Cert. No. NMv-5, was used) for 10–15 minutes.

5. Rinse in running tap water.

6. Rinse for 5 seconds in a mixture of equal parts of 95% ethyl alcohol, methyl cellosolve and tertiary butyl alcohol.

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[2]Johansen, D. A. 1938. Tertiary Butyl Alcohol methods. IV. Hygrobutol methods. El Palo Alto News 2: 1–4.

7. Immerse for 10 minutes in a fast green FCF solution prepared as follows: take one part of a saturated solution of the dye in equal parts of clove oil and methyl cellosolve, 3 parts 95% ethyl alcohol, 3 parts tertiary butyl alcohol, and add 3–4 drops of glacial acetic acid to each 100 cc. of solution. The dye (a Coleman and Bell product, purchased before certification was instituted, was used) should be dissolved in the solvent several days before being used; fast green usually dissolves slowly—an iridescent streaming first occurs and solution is not complete until this streaming has disappeared. If the acetic acid is omitted, the dye will be completely removed during succeeding stages. The time may require some adjustment according to the nature of the tissues and the fixing fluid. That cited is for formalin-aceto-alcohol and Navashin fixation; incidentally, the writer has found that the substitution of propionic acid for the acetic acid in these two fixing solutions gives far superior fixation.

8. Rinse for 5 seconds in a mixture of equal parts of 95% ethyl alcohol, methyl cellosolve and tertiary butyl alcohol plus a trace of acetic acid.

9. Immerse for about 5 minutes in the orange G stain. First prepare a saturated solution of the dye in equal parts of clove oil and methyl cellosolve, then take one part of this mixture and one part each of methyl cellosolve and 95% ethyl alcohol. (The dye originally used was a National Aniline product, Cert. No. NO-3.)

10. Rinse briefly in a wash composed of equal portions of clove oil, methyl cellosolve and 95% ethyl alcohol.

11. Rinse in a wash made up of equal parts of clove oil, absolute ethyl alcohol and xylol. (If Nevillite V is used for mounting, substitute toluol for the xylol.)

12. Rinse in xylol (or toluol) plus a trace of absolute ethyl alcohol.

13. Rinse in two changes of pure xylol (or toluol), then mount in dammar balsam, Nevillite V or other preferred medium.

The staining solutions can accommodate upwards of 500 slides (when held in Coplin jars) before replacement is required, but the washing solutions in steps 6, 8 and 10 will need changing frequently —usually after about every 50 slides. As soon as one of them becomes too heavily saturated with dye, it should be renewed; otherwise the sharpness of the stains will be diminished.

The slides when taken out of the first three staining solutions will be rather dirty and no differentiation is apparent, but when removed from the wash in step 10, they will be perfectly clean, provided Haupt's adhesive has been used for affixing the sections. If the periods as cited for each staining solution are adhered to, there is no

need for the exercise of personal judgment; differentiation is practically automatic. The staining process is continuous once the slides have been removed from the safranin.

The staining effects are little short of astonishing. Dividing chromatin is red, resting chromatin purplish; the nucleoli are usually red, occasionally violet, but in *Lilium* megagametophytes, which are exasperatingly difficult to stain adequately, they are a bright green. Nucleoplasm is colorless or greenish. Spindle fibers are purple. Lignified cell walls are a bright red, cutinized cell walls reddish purple, suberized walls red, cellulose walls greenish orange, and the middle lamellae green. Sclereids are a brilliant red; collenchyma cells are a bright green, or yellowish green when not fully developed. The cytoplasm is normally a bright orange, but when the combination is used on roots or rhizomes showing the origin of lateral roots, the cytoplasm of the latter is a dull green and the nuclei are purplish. Starch grains have a deep purple center with a green, and then an orange, halo at the periphery, but the two latter colors are unfortunately somewhat fugitive and become bleached or replaced by the purple. Plastids are yellowish-green to bright green. The callose portion of the guard cells of stomata should be bright red and the remainder of the cell wall purplish. Casparian strips are dark red and the rest of the cell walls of the endodermis yellow. Invading fungal mycelium is bright green; the combination, in fact, is unusually good for sections of the teliosori and uredosori of *Puccinia*. Sections of lichens take an excellent stain, and the algal and the various fungal regions are well differentiated. Since only killing fluids giving an acid fixation image have been used, the staining reactions of mitochondria are not known.

DELAFIELD'S HEMATOXYLIN AND SAFRANIN FOR STAINING MERISTEMATIC TISSUES

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ABSTRACT.—**The following technic is suggested for staining permanent preparations of meristematic tissues: Prepare and mount the sections by the usual paraffin method. From water, stain them 2-10 minutes in a solution made by adding 2-4 cc. of Delafield's hematoxylin to a Coplin jar full of tap water. As staining is progressive, the sections should be examined from time to time with a microscope. When the cell walls have become a deep purple, transfer the preparations, thru the usual series, to a mixture of xylol-absolute-alcohol in equal parts, and from this to a counterstain made by adding 4-6 cc. of a saturated solution of safranin in absolute alcohol to a Coplin jar full of xylol (75%) with absolute alcohol (25%). This stains the nuclei. Leave the sections in the counterstain at least 2 hours and then rinse them in xylol-absolute-alcohol (1:1) to remove excess safranin. Transfer them to pure xylol and then mount them in neutral balsam.**

The recent revival of interest in histogenetic problems in plants has created a need for better methods of staining the cell walls and nuclei in meristematic tissues. Altho several excellent technics have been devised for this purpose (Foster, 1934; Cross, 1937), the differences in the staining reactions of different species, and of a given species when different fixatives are used, are such that a universal method seems very remote. Difficulties encountered in obtaining satisfactory preparations of the shoot apex in certain species of *Acacia* (Boke, 1939) stimulated experimentation leading to a technic which involves the use of Delafield's hematoxylin and safranin. The present method of applying these well-known stains has yielded such excellent results with *Acacia*, *Ginkgo*, *Tradescantia*, and *Zamia*, that it seems worth while to call it to the attention of investigators who are interested in histogenetic problems. Moreover, it requires less time than the conventional methods.

Altho the best results were obtained when Juel's reagent (prepared according to a formula given by Korody, 1937) was used as a fixing medium, other good preservatives may be used. Sections are prepared by the usual paraffin method and mounted on slides. The following is a provisional schedule for staining, which may be modified according to the needs of the user:

1. Xylol—5 to 8 minutes.
2. 50% xylol–50% absolute alcohol—2 minutes.
3. Absolute alcohol—2 minutes.
4. 95% alcohol—2 minutes.
5. 70% alcohol—2 minutes.
6. 50% alcohol—2 minutes.
7. Water—2 minutes. Rinse several times.
8. Delafield's hematoxylin—2–10 minutes [prepared by adding 2–4 cc. of a stock solution, made according to a formula given by Chamberlain (1932), to a Coplin jar full of tap water]. This stains the cell walls an intense purple. Since there are always two variables, the concentration of the staining medium and the material being stained, the precise time must be determined by trial. Staining is progressive, however, and gradual enough so that the preparations may be examined from time to time with the aid of a microscope; this permits the intensity to be easily regulated. Depending upon the composition of the tap water used in its preparation, the staining medium is more or less stable. Its staining powers gradually decrease, owing to a deposition of pigment on the walls of the container. It has been found, however, to remain usable for two weeks or more.
9. Water—2 minutes. Rinse several times.
10. 70% alcohol—2 minutes.
11. 95% alcohol—2 minutes.
12. Absolute alcohol—2 minutes.
13. 50% xylol–50% absolute alcohol—2 minutes.
14. Safranin—2 hours (prepared by adding 4–6 cc. of a saturated solution of safranin in absolute alcohol to a Coplin jar full of a mixture of 75% xylol and 25% absolute alcohol. The resulting staining solution should be a deep red, but clear. If a precipitate forms, it should be filtered before using; it may be kept stable by the occasional addition of a few cc. of absolute alcohol to replace that which evaporates.) This differentiates the nuclei. With some materials, a period longer than 2 hours may be an improvement. As far as could be determined, meristem sections may be left in this medium indefinitely without being damaged, since nuclei do not overstain in it. If mature tissues occur in the material, lignins and tannins tend to retain too much safranin when the time is lengthened.
15. Destain in a mixture of xylol and absolute alcohol. Usually, a few quick rinses in 50% xylol in absolute alcohol will suffice. However, since the rapidity and extent of destaining depends largely upon the amount of alcohol in the destaining medium, the user may adjust this as he wishes.

16. Xylol—5 minutes.
17. Mount in neutral balsam.

Since the nuclei of many plants stain with Delafield's hematoxylin, the use of safranin as a counterstain may frequently be omitted. In this case, the time required for the entire staining process is shortened to less than an hour. It seems, however, that the safranin has a slight tendency to reduce the hematoxylin which is retained by the nuclei, so that the use of the counterstain may yield a preparation with more desirable contrasts. If the tissues do not readily take either the safranin or the hematoxylin, the use of a 1% solution of potassium permanganate as a mordant for 5 minutes previous to staining is suggested (Chamberlain, 1932).

Altho this method of using Delafield's hematoxylin has received mention in the literature (Chamberlain, 1932; Lee, 1937, pp. 159, 652), its value appears to have been underestimated. The method of making up the safranin solution was suggested by a similar procedure used by E. J. Kraus (described by Conant, 1926) who, however, used gentian violet instead of safranin. Cross (1937) and Jones (1939) have likewise used xylol in making up biological stains.

The advantage of the combination is that the preparations are excellent, both for visual work and for photographic purposes. Very satisfactory photomicrographs may be obtained by using panchromatic film and a light green filter.

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DIFFERENTIAL STAINING OF THE ANTERIOR PITUITARY GLAND OF THE CAT

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ABSTRACT.—**The terms "acidophile" and "basophile" as applied to the two recognized types of chromophilic cells of the anterior pituitary have been sanctioned by long usage. Nevertheless, these cells do not always give a specific reaction to acid and basic dyes respectively. The difficulty of classification has been further complicated by the demonstration of an additional tinctorial type in the female rabbit and cat which has been provisionally classed as a modified acidophile. This distinction is based on the differential staining reaction obtained with Heidenhain's "azan" technic following sublimate-formalin fixation, the standard acidophile reacting with orange G, the modified type with azocarmine.**

Both of these cells are stained with copper hematoxylin, Heidenhain's iron hematoxylin, Mallory's phosphotungstic acid hematoxylin and Kultschitzky's acetic hematoxylin. However, Hansen's chromalum hematoxylin stains the basophiles intensely and also reacts very slightly with the so-called modified acidophile.

The orange G + phosphomolybdic acid + hematoxylin method of Hall and Hunt stains the basophiles more or less selectively when applied progressively, altho the special cells also react. When regressive staining is employed, the results vary depending on the fluid used. Following successful differentiation with Weigert's borax ferricyanide, the basophiles are decolorized and the modified acidophiles are deep blue, while the ordinary acidophiles retain the orange G. These staining reactions are unmodified after Bouin's fixation.

It is concluded that the weight of evidence favors the classification of the special cell as a modified type of acidophile.

INTRODUCTION

The continued interest in the problem of the differential staining of the cellular components of the anterior pituitary is evidenced by the fact that three methods have been described in this journal (Koneff 1938, Maxwell 1938, and Lewis and Miller 1938), since Dawson and Friedgood (1938a) described a technic which differentiated the acidophile of the female rabbit and cat into two distinct tinctorial types. In this method the glands were fixed in sublimate-formol followed by Heidenhain's "azan" modification of Mallory's connective tissue stain. The ordinary or standard acidophile was

colored orange, while the cell which is present during the sexual activity of the female rabbit and cat appeared a deep red due to its special affinity for azocarmine. Material based on this method has been described and demonstrated (Friedgood and Dawson 1938, and Dawson and Friedgood 1937, 1938b).

It has been suggested that the affinity for azocarmine was due to the greater residual mitochondrial content of the cells, since sublimate-formol does fix mitochondria, altho not as well as other fixatives especially designed for this purpose. Others felt that the 'carmine' cell might possibly be an atypically stained basophile. It seemed well to investigate these two possibilities. A variety of staining procedures has been tried on the pituitary of the cat, usually after sublimate-formol fixation, but in one instance Bouin's fluid was used. The relatively high content of acetic acid in this mixture usually destroys the mitochondria, altho this does not always occur.

Specificity of Acid and Basic Stains

The terms 'acidophile' and 'basophile' as applied to the two chromophile cells of the anterior pituitary are sanctioned by usage and probably derived originally from the appearance of these cells after routine staining with one of the common hematoxylins and eosin. Many authors, recognizing that the staining reactions, especially with the anilin dyes, were not always consistent with this earlier distinction of "acidophilic" and "basophilic" types, have preferred to designate the cells by letter or by number so that the implication of a reaction with a specific category of stain could be obviated. It seemed immaterial what system of designation was employed so long as investigators were dealing with well-recognized cell types, but when the possible existence of another type of chromophile is suggested, difficulties of classification immediately appear.

The size and nature of the granules which react with the dyes may also be used to supplement the information obtained by differential staining, but in many instances the appearance of the granulation varies with the fixing fluid used.

Maurer and Lewis (1922) found that the so-called acidophilic cells were stained by safranin, a basic dye, but in the method of Severinghaus, the basophilic cells are stained by methyl green, a basic anilin dye, while in the method of Cleveland and Wolfe (1932) erythrosin, an acid dye, may stain one variety of basophilic cell.

Many of the special hematoxylin stains also behave in an apparently inconsistent manner. Rasmussen's (1936) report, that copper hematoxylin specifically stains the acidophiles but does not stain the basophiles as stated by MacCallum, has been completely

confirmed by Severinghaus (1937) and his co-workers. More recently Hall and Hunt (1938) have described a method employing a simple aqueous hematoxylin, following phosphomolybdic-orange-G and phosphomolybdic acid, which stains one variety of basophile intensely. Warbritton and McKenzie (1937) were fully aware of the inconsistent results obtained by varying fixation and staining methods, and have made a rather complete analysis of the results obtained, but were unable to materially clarify the problem.

The difficulty, moreover, is not confined to the pituitary gland but has its counterpart in other tissues as well. Crossman (1939) has attempted to lay down procedures which will enable the histologist to separate the acidophilic elements of tissues into two groups, based on relative intensity of the acidophilic reaction. Furthermore, similar difficulties have been encountered in finding a stain which is specific for chromatin, and at present chromatin is best identified by a microchemical test (Feulgen's reaction) rather than on the basis of specific staining by recognized basic stains.

Experimental Staining

We may now return to the problem of the basis for recognition of two classes of acidophiles in the cat pituitary or perhaps to the question as to whether the cell type under discussion may be a basophile. The "azan" modification of Mallory's method does not employ a basic dye, and the orange G and anilin blue are sulphonated. After staining with azocarmine, and before differentiation, the special cell is a deep, dark red, the ordinary acidophile, a light red and the dense basophiles, a brilliant red. After differentiation and staining with orange G + anilin blue, only the special cell retains the azocarmine, the ordinary acidophiles are orange and the basophiles blue. When azocarmine staining is omitted both the special cell and ordinary acidophile are orange. However, if anilin blue is present above the optimum concentration, the ordinary acidophile may appear brown due to the addition of anilin blue to the orange G already present. With this combination of stains, so-called selective staining is present to some degree but is relative rather than absolute. However, the final results under a properly controlled procedure are consistent and three selectively-stained cell types are obtained. Nevertheless, the ordinary acidophile may show some affinity for anilin blue and the basophiles first react strongly with azocarmine which is subsequently replaced with anilin blue.

After establishing the tinctorial characteristics of these cells which may also be recognized by differences in form, size, and type of granulation, a variety of hematoxylin stains was applied. With

copper hematoxylin both the special cell and the ordinary acidophile are intensely stained. The special cell is a dull blue, the ordinary acidophile a deep clear blue and the basophiles appear light brown to yellow flecked with incompletely stained mitochondria.

The results obtained by the method of Hall and Hunt are variable depending on the mode of staining. With preliminary staining in orange G followed by a minimum staining in the hematoxylin (3 minutes) without differentiation, the ordinary acidophiles are dull orange, the special cell light to dark brown and the basophiles purple-blue to light blue depending on the degree of their granulation. Both the special cell and the ordinary acidophile tend to react with the hematoxylin but the special cells have a much stronger affinity for this stain. This picture is obtained only if dehydration is rapidly accomplished by passing the slide directly to 95% and absolute alcohol. If dehydration is done slowly thru a graded series of alcohols, the three classes of chromophile cells are a uniform blue color. When the staining in the hematoxylin is more prolonged and differentiation is attempted, the picture changes rapidly. With acid alcohol the blue color is quickly extracted from the basophiles and both the special cell and ordinary acidophile are stained a deep blue. If Weigert's borax-ferricyanide is used for differentiation, the blue color is usually more or less completely extracted from the basophiles and the special cells become a deep blue while the ordinary acidophiles remain orange or light brown. Differentiation with Weigert's fluid is difficult to control and uniform results are not always obtained, but when successful, the differentiation between the special cell and ordinary acidophiles is very striking.

Heidenhain's hematoxylin, Mallory's phosphotungstic acid hematoxylin and Kultschitzky's acetic hematoxylin stain both the special cell and the ordinary acidophile characteristically and with almost equal intensity. Occasionally slight differences in the tinctorial quality of the two cells may be recognized. With Kultschitzky's hematoxylin, when orange G is used as a preliminary stain according to the method of Hall and Hunt (1938), the orange G is replaced but a greenish cast attests to the presence of masked orange G. The basophiles are not stained by these methods. Hansen's chromalum hematoxylin (Bensley and Bensley 1938), if used alone, stains the basophiles a deep blue to purple, while the ordinary acidophiles are colorless and the special cells slightly tinted with blue. When eosin is used as a counterstain, the ordinary acidophiles are brilliant red and the special cells a dull red due to their slight reaction with the hematoxylin.

In order to test the possible effect of the staining of residual mitochondrial material following sublimate-formol fixation, the pituitary gland was also fixed in Bouin's fluid. The azan modification of Mallory's method gave a differential staining similar to that following the sublimate-formol fixation. If anything, the contrast between the azocarmine-stained special cell and the orange-G-stained ordinary acidophile was enhanced—a result similar to that obtained in the rabbit pituitary following fixation in formalin alone. Bouin's fixation, however, like formalin alone, fails to fix the basophiles and chromophobes properly. Accordingly the azocarmine reaction of the special cells cannot be attributed to their greater mitochondrial content; this reaction further indicates that the differential staining with azocarmine and orange G is due to specific differences in the granules of these cells, as revealed by a fixing fluid which contains a relatively high proportion of formalin without bichromate. Furthermore, Bouin's fixation does not modify the staining reactions obtained with the several hematoxylins employed following sublimate-formalin fixation.

The cumulative evidence from differential staining shows clearly that the special azocarmine cell is quite unlike either the standard acidophile or basophile, and its characteristic behavior during the reproductive cycle of the cat affords strong confirmation of its functional independence, altho the significance of its presence and activity is hypothetical at present (Dawson and Friedgood, unpublished). The cell apparently arises from a chromophobe and, when degranulated, returns to a chromophobic state. Moreover, no transition between this cell and the other chromophilic cells has been observed. Whether the special cell is to be classed as an acidophile or basophile is dependent on the relative emphasis placed on the unique specificity of the several stains which have been employed to demonstrate the two classes of chromophiles. My strong inclination, even in the face of some conflicting evidence, as given above, is to regard it as a variety of acidophile.

The suggestion of recognizing a third type of granular cell in the anterior pituitary is regarded by some authors as introducing an unnecessary confusion into a situation which is already too complicated. It also seems unwise to beg the question, however, in the face of the evidence at hand. In many ways there is a close parallel between the cell under discussion and the so-called 'pregnancy cell' which Severinghaus (1937) feels should be regarded as an acidophile in high secretory activity altho it develops from the chromophobes as pregnancy ensues, remains in an active secretory phase thruout

pregnancy and reverts again to the chromophobic state some time after parturition. [Altho these cells are relatively numerous in certain periods of gestation as well as in early postpartum, they cannot be regarded as "pregnancy cells" since they are also found in limited numbers in normal and castrate males, and in proestrous, estrous and castrate females, being typically absent only from anestrous animals.] At the present no final conclusion can be reached as to the nature of the granular content of the azocarmine cell and the matter will have to await the physiological assay of the pituitary gland when these cells are present in great numbers as in late pregnancy.

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PRESERVATION OF TRYPAN BLUE AND NEUTRAL RED WITHIN THE CELLS OF LOOSE CONNECTIVE TISSUE

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ABSTRACT.—**Trypan blue granules within the histiocytes and neutral red stained granules within the mast cells of the same areolar tissue spread can be preserved in their original colored state by the following method: fix in 10% formalin for 12-24 hours; rinse in distilled water; place in two changes of dioxan for 5-10 minutes each (overnight in second change will not hurt); mount from the second dioxan using diaphane dissolved in dioxan as a mounting medium, or clear in xylene and mount in balsam. No dye is lost, and no cellular distortion occurs. Fast green is used as a counterstain (1% aqueous plus 0.2% acetic acid). Stain ½ to 1 minute and rinse.**

INTRODUCTION

This method was devised to make permanent preparations of vitally stained areolar tissue spreads, illustrating both intravital staining by trypan blue and supravital staining by neutral red upon a single slide. A survey of the literature shows that various methods have been employed to preserve each of these dyes when used separately. A critical analysis of the methods used for the fixation of trypan blue was given by Hetherington and Tompkins (1933). They claim that 10% formalin, as used by many investigators, is unsatisfactory when minute amounts of dye must be detected, altho it preserves the cell contours and type of dye deposition. They found that the fixing fluid of Carnoy and Lebrun gave perfect preservation of the dye, but crystals of mercuric chloride caused some interference. Baird (1935) found that 5% neutral formalin followed by acetone dehydration satisfactorily preserved trypan blue in subcutaneous tissue spreads.

Neutral red is more difficult to preserve satisfactorily. A survey of methods used was given by Cunningham and Conn (1932). Following the example set by McJunkin (1925), Zenker-formol has been used extensively as a fixative for neutral red and similar dyes. The problem of dehydration without the use of alcohols was solved by McJunkin and others by employing acetone, followed by benzene or xylene. Hu (1931) found that 100% alcohol, to which $HgCl_2$ and neutral red had been added, permitted complete dehydration without any loss of neutral red from the cells. This method had the further

advantage that paraffin sections of tissue vitally stained by neutral red could be easily counterstained if the dye solution also contained $HgCl_2$ and neutral red. Morrison, Gardner, and Reeves (1936) preserved neutral red in paraffin sections of gastric mucosa by "Susa" fixation, followed by 90% and 100% alcohol, clearing in carbon disulphide, and paraffin embedding. When films can be dried, no dehydration problem is encountered.

Procedure

Modifications of the foregoing methods were tried in order to preserve both trypan blue and neutral red in the same spread of tissue in as nearly as possible their original appearance in the freshly made spread. Various counterstains were tried in order to bring out the

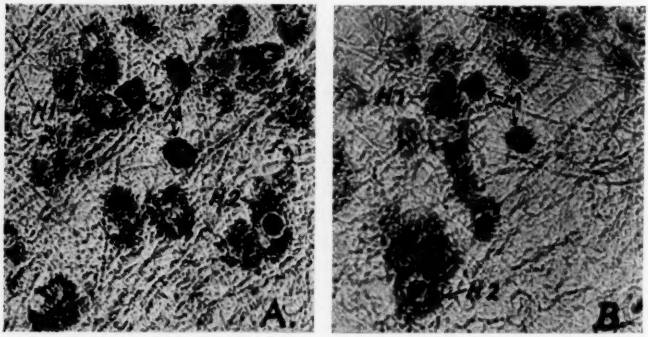

Fig. 1. Identical cells from an areolar tissue spread from a vitally stained white rat, before fixation (A), and after fixation, dehydration, and mounting (B). M, mast cells (neutral red); H1, histiocyte showing trypan blue granules; H2 histiocyte with engorged leucocyte. × 268.

fibrocytes and other tissue elements without altering the vital staining of the histiocytes and mast cells.

A single subcutaneous injection of 2.5 to 3 cc. of a fresh sterile 1% aqueous solution of vital trypan blue (Coleman and Bell Co.) for rats weighing 85–100 g. (5 cc. for mature rats) was sufficient to mark the histiocytes 48 hours after injection. Following light etherization, the animals were killed by severing a large artery. The neutral red solution was injected directly into the subcutaneous areolar tissues of the groin, the needle being directed so as to make several dye deposits around the original puncture. The sample used was certified (NX 5) neutral red, Ehrlich, (79% dye content, National Aniline) 0.02% in 0.9% NaCl. In 3–5 minutes an incision was made and small blobs of the edematous tissue were removed.

placed on clean slides, and teased into thin spreads by the use of needles and small pieces of filter paper. When the corners were dry and attached to the slide, the spread was ready for fresh observation or for fixation.

The most brilliant results were obtained when 10% formalin was used as the fixative. The slight acidity of the commercial formalin is desirable because neutral red acts as an indicator, being red in acid and orange or yellow in alkaline solutions. The 10% formalin should be tested by adding to it a drop or two of neutral red and observing any color change. If it turns orange, cautiously add $N/10$ HCl until it turns red. Fixation is complete in a few hours, but it has been my practice to let the slides fix overnight. Several days fixation in formalin will not hurt the dyes.

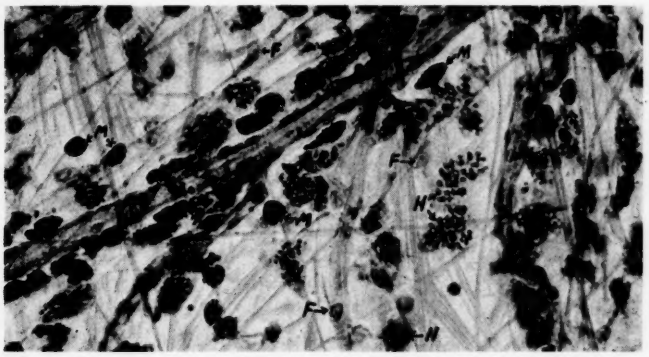

FIG. 2. Permanent areolar tissue spread from white rat intravitally stained by trypan blue, supravitally stained by neutral red, and counterstained by fast green. M, mast cells (neutral red); H, histiocytes (trypan blue); F, fibrocytes (fast green). × 268.

The slides are first rinsed in distilled water and then placed in two successive changes of dioxan (Coplin jars 1 and 2), 3–5 minutes each depending on the thickness of the spread and the amount of agitation. They may stay in the second change indefinitely. The slides are mounted directly from dioxan, using as a medium hardened diaphane (Will Corp.) redissolved in dioxan. They may also be placed in xylene and mounted in balsam. The blue granules within the histiocytes (macrophages) and the fine red granules of the mast cells have very near the same color values as they had in the fresh spreads.

Zenker-formol (85 parts stock plus 15 parts formalin) also preserves the dyes well, altho they will have slightly different color values. $HgCl_2$ crystals will be present to some degree, but 24 hours in dioxan will remove a large proportion of them.

The most useful counterstain tried was fast green F. C. F. (Warner-Jenkinson Co., dye content 85%). A 1% solution in distilled water plus 0.2% glacial acetic acid for ½–1 minute following the rinse after fixation brings out the nuclei, cytoplasmic processes, fibrocytes and fibers. See fig. 2. After staining, rinse in distilled water and place in dioxan.

A few drops (¼ cc.) of Delafield's hematoxylin added to 100 cc. of the 10% formalin fixing fluid stains the nuclei of the spread in 24 hours without interfering with the vital staining. The cytoplasm and fibers of the spread can then be stained by the use of 0.5% aqueous erythrosin (cert. no. CEr-2 Coleman and Bell) for 1 minute.

Summary

By the above methods there is no appreciable loss of either trypan blue or neutral red following fixation, clearing, and mounting. Figure 1, A and B, shows the same cells before fixation, and after mounting. The three mast cells (M) have retained their relative positions, but the histiocytes have migrated, and also do not appear in the same focal plane in fig. 1, B that they had in fig. 1, A. By comparison of the trypan blue granules of histiocyte 1 (H1) in each figure, it is evident that all of the dye is retained.

Slides made four years ago by essentially the same process have retained both dyes without any fading.

The most useful technic employing a counterstain is as follows (see fig. 2):

1. Fixation—10% commercial formalin, 12–24 hours.
2. Rinse in distilled water, 1–2 minutes.
3. 1% fast green plus 0.2% glacial acetic acid, ½–1 minute.
4. Rinse in distilled water, 1 minute.
5. Dioxan 1., 5 minutes.
6. Dioxan 2., 5 minutes.
7. Mount in diaphane dissolved in dioxan.

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A STAINING METHOD FOR THE ANTERIOR HYPOPHYSIS OF THE RAT

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Abstract.—**A staining procedure for the anterior hypophysis of the rat, differentiating between eosinophilic granules, basophilic granules and mitochondria, has been divised. Small pieces of hypophyseal tissue are fixed in Champy's fluid. Following fixation the tissue is either chromated or osmicated. After being embedded in 60–62° paraffin, the tissue is cut serially at 2 and 3 μ. The sections are stained with 7% Altmann's acid fuchsin by heating on a laboratory hot plate, followed by 30 seconds in a 2% solution of Orange G made up in 1% phosphomolybdic acid. They are then treated for 10 seconds in a .01% solution of potassium carbonate, and stained for 10–30 minutes in Goodpasture's acid polychrome methylene blue. The mitochondria stain brilliant fuchsin, the eosinophilic granules orange-red, and the basophilic granules deep blue.**

During the course of recent studies on the anterior hypophysis of the rat, a staining procedure was evolved which satisfactorily differentiates between basophilic granules, eosinophilic granules and mitochondria. The method is somewhat similar to the technics of Severinghaus (1932) and Volkonsky (1932) and was arrived at by making successive modifications of these two technics.

The pituitaries were fixed in Champy's fluid (2 parts of 1% chromic acid, 2 parts of 3% potassium dichromate, and 1 part of 2% osmic acid). Glands weighing up to approximately 10 mg. were divided sagitally into two pieces before fixation; larger glands were usually divided into three or more pieces. In the post-fixation treatment, dehydrating and embedding of the tissue, the methods[1] of Severinghaus (1932) were followed in detail. A majority of the tissue was chromated following fixation (modified Champy-Kull method of Severinghaus). Tissue which was to be used for study of the Golgi apparatus was osmicated following fixation (Nassanov-Kotatchev technic). All tissue was embedded in 60–62° paraffin. Serial sections were cut at 2 and 3 μ; the latter took a more intense stain.

After the sections were run down to water, they were treated as follows:

[1]More detailed directions for the post-fixation treatment and embedding of tissue were kindly furnished us by Dr. Severinghaus in a personal communication.

1. The sections were covered with 1 cc. of 7% Altmann's acid fuchsin. This amount was applied with a pipette so that it covered the lateral two-thirds of the slide. The sections were then heated on a rheostat-controlled laboratory hot plate. In some instances the sections were gently heated to steaming 1–3 times with one minute intervals between the steamings. In most instances less intense heating, without the production of steam, gave better results. Since the hot plate was controlled by a rheostat, it was so regulated that it was hot at one end and cool at the other. Therefore, it was possible to place the slide at different positions to obtain the amounts of heat necessary. The slides were placed on the hot plate at a point where one could barely hold the thumb. They were heated four times for 15 seconds each with a 45 second interval between heatings and a final cooling period of 5 minutes after the last heating. We have found that the reaction of the tissue to acid fuchsin varies from gland to gland and the optimal staining time must be determined for each gland.

2. The acid fuchsin was drained off and the slide rinsed with distilled water. The section was then stained for 30 seconds in a 2% solution of Orange G made up in a 1% solution of phosphomolybdic acid.

3. The slide was rinsed in distilled water and then immersed for 10 seconds in a 0.01% solution of potassium carbonate. The fluid was agitated by moving the slide.

4. The slide was rinsed quickly in distilled water and stained for 10–30 minutes (usually 12 minutes) with Goodpasture's acid polychrome methylene blue (1917). Good results were also obtained by staining in Masson's aniline blue (1928) for one hour. The sections were rinsed in distilled water, passed thru 95 and 100% alcohols, cleared and mounted in balsam.

In sections stained by this method, the mitochondria stained brilliant fuchsin, the eosinophilic granules orange-red, and the basophilic granules deep blue. In sections to which a less intense fuchsin stain was applied, the mitochondria took a lighter fuchsin, the eosinophilic granules orange or yellow-orange, and the basophilic granules a deep blue. The connective tissue usually stained blue while the red blood cells stained either fuchsin or orange, or intermediate between these two.

If it was found necessary, the sections intended for Golgi preparations were bleached before staining. The sections were flooded with 0.125% solution of potassium permanganate for approximately 30 seconds; they were rinsed in distilled water and were treated with a

1% solution of oxalic acid for 30 seconds. They were then rinsed in distilled water and stained by the procedures described above.

This staining procedure differs chiefly from those of Severinghaus and Volkonsky in that differentiating solutions such as picric acid and aurantia were not used to bring about the differentiation between the eosinophilic granules and mitochondria. It seems that, in tissue fixed in Champy's fluid and handled by the methods indicated above, there is a certain inherent difference between the mitochondria and the eosinophilic granules in their capacity to react to certain stains. This difference apparently makes it possible for them to be stained selectively without the use of the differentiating fluids mentioned above. The success of this procedure in differentiating the eosinophilic granules and mitochondria seems to depend on obtaining a fuchsin stain of just the optimal intensity. This depends on the heat of the hot plate at the spot where the slide is placed, the length of the heating periods and the cooling times allowed. Finally, the amount of stain applied to the slide and the area covered are probably important since these factors determine the thickness of the layer of fluid which in turn determines the rapidity with which the solution will heat. If, on the other hand, the sections are overstained in fuchsin, satisfactory differentiation between the eosinophilic granules and the mitochondria will not be obtained. We have, however, found it comparatively easy to arrive quickly at the correct intensity of the fuchsin stain.

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ROOT-TIP SMEAR METHOD FOR DIFFICULT MATERIAL

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Abstract.—**A technic is outlined for the preparation of difficult material for the study of chromosome number and morphology in root-tip smears. The chief objective is to obtain polar views of the metaphase plates rather than equatorial views. To achieve this end it is recommended that fresh root-tips be cut free-hand into thin cross sections. The important features are: *thin freehand cross sectioning* of the *fresh root-tips;* fixing in Belling's iron aceto-carmine solution; maceration for 2–5 minutes in 50% HCl in 95% alcohol; and mounting in "Diaphane."**

The writer has from time to time successfully used several of the published root-tip smear methods. Occasionally, however, material has been encountered for which none of the ordinary methods proved satisfactory. Since the spindles of the division figures are usually oriented in the direction of the axis of the root, the smearing of either fairly large pieces of root-tip or small longitudinal strips, recommended in previous methods, results almost exclusively in equatorial views of the metaphase plate. Their success or failure depends on one's ability to diagnose such figures when flattened. Where the cell volume is large and the chromosome number relatively low this is not difficult, but in material where the cell volume is small and the chromosomes numerous, they are usually so entangled in a flattened equatorial view as to make accurate diagnosis uncertain or impossible.

To increase the proportion of polar views the following method has proven useful. After the fresh root-tips have been thoroly scrubbed free of sand and soil, they are cut into the thinnest possible cross sections, free-hand, with a sharp razor blade. With practice and care in this operation, sections will be obtained which are not more than two or three cells thick, and these are selected for mounting. In several cases this additional procedure prior to fixing has yielded results where previous methods failed (see Fig. 1). Recently, however, while endeavoring to obtain counts on tetraploid *Lactuca* species, it was found that the chromosomes were so excessively long in relation to the diameter of the nucleus that polar views could not be counted with facility. In this case the difficulty was overcome by soaking excised tips in a 0.01% solution of colchicine for 2 or 3 hours, after the method of O'Mara (1939), before sectioning and fixing.

This drug acts as a prefixation agent and greatly shortens the chromosomes increasing the clarity of the metaphase plates.

After fixing, digestion in HCl softens the material and aids in flattening. In the Feulgen technic (Heitz, 1936) a *N* aqueous solution of HCl is used for another purpose, and simultaneously works to this end. For use with aceto-carmine, a mixture of equal parts 95% alcohol and concentrated HCl (Warmke, 1935) applied for 2–5 minutes is recommended.

In flattening, care should be taken to prevent any lateral movement of the cover, as this will turn the cells on their sides and thus defeat the purpose of the method. This can usually be avoided by placing a piece of filter paper on top of the slide, and while holding it firmly in place, gently stroke it from one end of the slide to the other. After the excess aceto-carmine has been removed in this way, the slide may

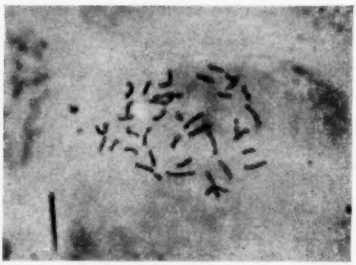

FIG. 1. Division figure from root-tip of tetraploid *Lactuca sativa* L. showing 36 chromosomes (not treated with colchicine before fixation). × 890.

be warmed slightly, and the final flattening done by pressing lightly on the cover with a needle. In making the preparation permanent, the cover is floated off in a mixture of equal parts of absolute alcohol and acetic acid, and is mounted directly in "Diaphane." This method involves the least manipulation and is therefore recommended.

The following is the complete schedule for aceto-carmine smears:

1. Where a preliminary trial indicates its necessity, washed, fresh, excised root-tips, ½ inch long, are soaked on 0.01% solution of colchicine for 2–3 hours. Otherwise omit and begin with step 2.
2. Cut fresh root-tips into the thinnest possible cross sections free-hand with razor blade.
3. With needle transfer sections to shallow vial or dish containing 2–3 drops of strong aceto-carmine. Fix for a few minutes or until very darkly stained.
4. Add several drops of 45% acetic acid to dilute the stain and

prevent overstaining. The material may be stored several days in this condition.

5. With a medicine dropper draw up a single slice and deposit on slide.

6. Holding the slide at an angle over a waste jar add, just above the slice, a drop of 50% concentrated HCl in 95% alcohol. As the first drop runs off, add more until all the aceto-carmine has been rinsed away.

7. Place the slide flat and keep material covered with additional drops of acid-alcohol for 2–5 minutes.

8. Rinse off the acid-alcohol in the same way using 95% alcohol.

9. After rinsing 2–3 minutes, dry around edges and add a drop of strong aceto-carmine.

10. After it has restained a minute or two, add a drop of 45% acetic acid and cover.

11. Blot up excess stain and flatten with heating.

12. To make permanent, float off cover in mixture of equal parts absolute alcohol and glacial acetic acid and mount in "Diaphane."

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NOTES ON TECHNIC

SUMAC WAX AS EMBEDDING MATERIAL IN BIOLOGICAL TECHNIC.[1] In an attempt to find a substitute for paraffin wax as embedding material for the preparation of microscopic slides, the wax from the seed of the lacquer tree (*Rhus sp.*) proves very satisfactory. Altho better known commercially as Japan wax, sumac wax is produced in large quantities in south-west China, being used in Kweichow, Szechuan and Yunnan for the making of soap and candles. When refined, it is a pale yellow solid with a melting point of 51° C. It is readily soluble in ether, xylol, chloroform, and castor oil; slightly soluble in warm ethyl alcohol; and insoluble in water and glycerin.

In order to test the suitability of sumac wax as an embedding material, *Lycopodium* (old strobilus), *Xylaria* (old stroma), *Auricularia*, bamboo (leaf), *Hydra*, and mouse pancreas were fixed in various fixatives (formalin, formalin-alcohol, chromo-acetic solution, or Bouin's fluid), dehydrated with alcohol, cleared in xylol or chloroform, and were finally embedded in sumac wax. The transfer of the materials from absolute alcohol to sumac wax thru chloroform may be accomplished within one day.

Sections of 1–3 μ in thickness may be made with ease on a sliding or rotary microtome. Thicker sections require careful handling due to the brittleness of the sumac wax. This brittleness is, however, to a large extent overcome by mixing 9 parts of sumac wax with 1 part of beeswax, or by mixing 3 parts of sumac wax with 7 parts of paraffin wax.

When stained with safranin and light green, safranin and gentian violet, Delafield's hematoxylin and eosin, or Haidenhain's hematoxylin, the finished preparations are in every way comparable to similar sections prepared with paraffin as the embedding material.

The only disadvantage of sumac wax compared with paraffin wax as an embedding material is its brittleness. But this may be easily removed by mixing it with a little beeswax or paraffin wax as pointed out above. The advantages of using sumac wax are: its comparatively low cost in China; the readiness with which thin sections may be obtained; the fact that it can stand a relatively high temperature (60° C.) without spoiling the material embedded in it; and the readiness with which it may be dissolved in chloroform. The fact that it is soluble in castor oil offers a further advantage to countries where xylol is more expensive than castor oil, for the latter may be employed as a clearing agent in place of xylol.—J. Hsŭ and P. S. TANG, Physiological Lab., Tsing Hua Univ., Kunming, China.

[1]Aided by a grant from the British Boxer Indemnity Fund to J. Hsŭ.

CAUTIONS IN THE USE OF DIOXAN. Because of the frequent reference to the dioxan technic in recent papers in STAIN TECHNOLOGY, some of our readers have urged that a word of caution be included in our pages as to the possible toxic action of the chemical in question. It is generally agreed that dioxan has toxic properties; and recently Magruder[2] has thought it wise to publish a special warning on the subject, in a paper mentioning its use. He calls attention to reports that breathing only a small amount of its fumes can cause serious bronchial, liver or kidney disturbances, and states that its employment in some British laboratories has been forbidden because of its danger.

This question has been referred personally to Dr. Magruder by one of our correspondents; and in reply he states that too little experimental work has been done on the subject to justify a definite statement one way or the other as to the amount of danger involved in the use of this chemical. Presumably the danger depends on the amount of fumes inhaled; and probably there is little danger if the reagent is used in a well-ventilated room by a comparatively small number of people. When employed by a considerable number of students in a college class, Dr. Magruder recommends the following precautions: Keep dioxan containers closed (vaseline seal) when not in use. Have room well ventilated and air in circulation. Suggest that when changing tissue from one vessel to another, those containing dioxan be kept at some distance from the nose. Do not allow its use by any student who has ever had any suggestion of liver, kidney or lung trouble.

The fact that dioxan has now been employed for some time in many laboratories without any trouble seems to imply that it is not dangerous if handled with reasonable precautions. It is a valuable reagent, and should not needlessly be discarded; but the above-mentioned precautions should certainly be observed.—H. J. CONN, Geneva, N. Y.

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[2]Magruder, S. R. 1938. A comparative study of dehydration. J. Lab. and Clin. Med. **23**, 405–11. (See p. 411).

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

MICROSCOPE AND OTHER APPARATUS

ARDENNE, MANFRED VON. **Die Keilschnittmethode, ein Weg zur Herstellung von Mikrotomschnitten mit weniger als 10^{-3} mm. Starke für elektronenmikroskopische Zwecke.** *Zts. Wis. Mikr.* **56**, 8–23. 1939.

Maximum resolution and contrast with the electronmicroscope depend upon the thinness of the sections. To obtain paraffin sections of less than 1 μ in thickness, the author designed an auxiliary tilting device to be used in connection with the usual rotary microtome which permits the cutting of "wedge" sections.

Razor blade steel has been found satisfactory. By means of a special sharpening process the facet angle can be reduced to 10°, the cutting angle to 5°, and the radius of curvature at the edge of the 5 mm. knife is 10^{-4}. The knife is held by strong jaws adjustable by set screws and put in the place of a large knife. The object is prepared as follows: Lift off one layer from object to obtain a smooth surface; replace it by a covering layer of some equally hard substance such as paraffin of the same melting point which will not alter the surface. Apply it with a fine hair brush. Tilt the object slightly. The final cut produces a wedge shaped section. Dissolve away the cover. The tissue section allows easy further sectioning. The little roll of sections is placed on a drop of water at 40° C. to flatten out, and then treated as usual.

An ordinary object holder is attached to concave surface of a Leitz-Minot microtome by means of special springs and screws allowing the slightest tilt of the object to be controlled and registered. Tilting angles of less than 0.0001 are obtainable. One turn of the screw produces a 0.02° rotation.—*E. Barasch.*

HARDING, J. P. **A simple instrument for dissecting minute organisms.** *J. Royal Micro. Soc.*, **59**, 19–25. 1939.

The paper describes the construction of an inexpensive micromanipulator based on the principle of the pantograph, and an attachment for focusing the microscope without using the hands. It is suitable for dissecting Ostracods and Copepods. The micromanipulator consists of two brass dissectors mounted on a brass base attached to the stage of a dissecting microscope. Each dissector is equipped with a manipulating knob and a dissecting needle; dissecting needles are made from .05 mm. tungsten wire by honing or by burning the tip in a small flame; dissectors are attached to the base by a ball-and-socket joint made by fitting a bicycle ball-bearing in a socket drilled in the base. Each needle is controlled by moving its manipulating knob in the required direction instead of an arrangement of mill heads. The base has a piece cut out to accommodate a depression slide. The instrument may be adapted for use with an ordinary microscope by reversing the positions of needle and ball-and-socket joint. The attachment for focusing the instrument without using the hands consists of a lever fixed to the coarse adjustment head so that it projects over the edge of the table and is approximately horizontal when the microscope is in focus. A spring or rubber band connects the lever with the back of the microscope stage and tends to raise the head of the microscope out of focus; but this is counteracted by a rod attached to the end of the lever and operated by the raising or lowering of the worker's knee. Crustacea are dissected in benzyl alcohol which is reported to be superior to glycerine for this purpose. Unstained appendages may be mounted in Hyrax (Flatters & Garnett, London) because of its high refractive index.—*H. E. Finley.*

MICROTECHNIC IN GENERAL

ENBOM, G. **Ein Schnellverfahren zur Rekonstruktion in Plastilin nebst anderen technischen Beiträgen.** *Zts. Wis. Mikr.*, **55,** 150–65. 1938.

The author proposes to substitute modeling clay for the complicated wax method of reconstruction. He indicates how casts of the temporary model can be made with cotton soaked with gelatin, and how the level of a section can be recorded with wax pencils on thin metal wires.

Slice rolls of modeling clay (Tekniska fabriken Pilen, Asarum, Sweden) into thin slices, place between linen cloths, roll out into sheets 1 cm. thick. Fold, and roll again until surface is smooth. Thin sheets out to desired thickness. Project every 2nd or 3rd section onto the clay sheets on a paper-covered projection table with a fine needle ("Cito" of K. Rasche Co., Weissbach, Germany) applied vertically. Cut out series of 10 cross sections each, completing details and outline. Stack up in order on a tray. Remove notochord and spinal cord segments, and some of "negative". Use pieces of heated iron wire, 0.5 mm. thick. Cover with celluloid sheet. Follow a profile photograph when fitting sections. Remove celluloid sheet, and the rest of "negative". Remove desired amount of body wall. Smooth out surface, remove wires.

For casts, fill in one system at a time with a thin cotton coat, pour in hot conc. soln. of gelatin appropriately colored. Let dry before starting on next system. Brush over lightly with olive oil before casting. Connect dried systems with cotton and colorless gelatin. Remove modeling mass.—*E. Barasch.*

FISHBACK, H. R. and GREGERSEN, A. **A permanent method of labelling microscope slides.** *Amer. Jour. Clin. Path., Tech. Suppl.*, **3,** 134–6. 1939.

The blank end of the slide is wiped with ether and the data written on it with India ink. This is brushed lightly and quickly with clear lacquer applied with a camel's hair brush. For contrast the back of this end of the slide is covered with white lacquer.—*George H. Chapman.*

GOMORI, G. **A differential stain for cell types in the pancreatic islets.** *Amer. J. Path.*, **15,** 497–9. 1939.

The following procedure is recommended: Fix thin slices of tissue (2 mm. or thinner) in an aq. fixative such as formalin, Bouin's, Zenker's, Stieve's, or Zenker-formalin, or better still in Bouin's fluid in which half of the acetic acid is replaced by sulfosalicylic acid and then diluted with an equal part of water before use. Embed in paraffin. Oxidize the sections after deparaffinizing with a solution containing 0.3% each of $KMnO_4$ and H_2SO_4, rinse and decolorize with a 1 to 5% solution of $K_2S_2O_5$. Wash thoroly. Stain in a well ripened solution of chrom hematoxylin made by mixing a 1% solution of hematoxylin with equal parts of a 5% solution of chromium alum, ripened 1 or 2 days after adding a few drops of H_2SO_4 to each 100 cc. (The brand of hematoxylin is not stated.) Filter before use and stain 15 min. to 1 hr. under microscopic control. Rinse and differentiate in 1% alc. HCl. Rinse and counterstain with phloxine or ponceau de xylidine (sources not given) using the latter dye as a 0.5% solution containing 1% acetic acid. Any red or orange acid dye can be used. Rinse and differentiate in a 5% solution of phosphotungstic acid until the connective tissue is decolorized. Only the strongly oxyphilic structures, such as erythrocytes, muscle fibers, oxyphils, and alpha cells, remain stained. Cover in balsam.—*H. A. Davenport.*

LEVIN, E. **Photoaufnahmen mikroskopischer Präparate ohne Apparatur sowie eine einfache und billige Methode, um Literaturbelege zu gewinnen.** *Zts. Wis. Mikr.*, **55,** 470–1. 1938.

A simple method of making photographs of low magnification is outlined by the author. According to the stain selected, simple or panchromatic plates are used. Proceed as follows: Place the coverslip of the slide on the face side of the plate, black paper under the plate to prevent halation, and expose to direct light on the ground board of the enlarging camera. For outline drawings, etc., smaller slides may be printed on normal movie film, Leica size, and the negatives enlarged. In order to retouch the negative, place it in the negative holder of the enlarging camera and make a slightly enlarged negative.

In order to make a diapositive of a freshly prepared slide use "Agfa-direct-duplicate-film" whose pre-exposure allows the second exposure to produce a

regular inversion. Printing or enlarging now produces a positive which may be projected in 1–2 hr. For the reproduction of tables or diagrams in publications, make photographs on bromide paper. Insert a clean glass plate in the camera to press it flat. Use a longer exposure and print the paper negative exactly like a film. For shaded drawings use contrast paper, and for prints extra contrast paper. Even colored and half-tone drawings may thus be photographed. This procedure saves much time and expense.—*E. Barasch.*

LIESEGANG, R. E. **Zur Theorie der histologischen Versilberung.** *Zts. Wis. Mikr.*, **55**, 415–8. 1938.

The author discusses the compounds and conditions involved in the production of nascent sliver.

Nascent silver is not colloidal since the presence of silver nuclei does not cause it to settle out. Supersaturation seems to be responsible for the formation of silver ppt., the amount varying with the concentration of the reducing agents and the purity of the medium, or presence of nuclei hastening precipitation. The following procedure may be used to study these phenomena: Make a gel from 87 cc. of a 10% gelatin solution containing 13 cc. of a 10% $FeSO_4$ solution in a beaker. Cover with 2 cc. 20% aq. solution $AgNO_3$. Metallic silver bands, some white, some black, appear in a few days. Their clearness at the bottom is enhanced by a little H_2SO_4. A smaller concentration of $FeSO_4$ alters the nature of the bands. This may be due to a precipitation of Ag_2SO_4 instead of Ag. $AgNO_3$ does not produce bands in all concentrations. After dialyzing the gelatin, in the absence of silver nuclei, an enormous supersaturation may be obtained. The distance between the colored bands is proportional to the amount of supersaturation since it indicates the migratory capacity of the silver molecule. The shade or intensity of color gives no indication as to the amount of reducing metal; e.g., the highest and lowest in a series of $FeSO_4$ concentrations produce solid coloration of the gelatin mass, intermediate solutions producing lighter bands. These may occur in any of the different forms of silver: translucent yellow or orange, solid black, dark rust, or grayish white.—*E. Barasch.*

MILOVIDOV, P. **Notizen über Mikrotechnik. III. Die Anwendung der Azetokarmin-Methode für die Färbung von fixierten Mikrotomschnitten.** *Zts. Wis. Mikr.*, **56**, 67–9. 1939.

The distribution, morphology and structure of chromosomes in root tip smears and the meiotic stages of pollen mother cells may be brought out distinctly and with good preservation by a modification of the original azocarmin method. Proceed as follows: Cover the serial sections which lie on the slide, free from paraffin and washed with water, with a few drops of Schneider's azocarmin solution. Warm slightly for 1–2 min. over micro-burner. Cool, wash with water, transfer for a few minutes to 96% alcohol, then successively to abs. alcohol, xylol alcohol, and pure xylol. Embed immediately in Canada balsam. Do not use much albumen in attaching the section to the slide. The procedure is rapid, avoids artifacts, aids in the study of meioses, and even stains cell membranes well.—*E. Barasch.*

PITOT, A. **Une méthode rapide d'exploration des lames.** *Bull. d'Histol. Appl.* **15**, 77–8. 1938.

To avoid waste of time by mounting unnecessary sections, they may be solidified in Canada balsam and examined before mounting. To do this, after removing sections from the paraffin bath, cover with a thin coat of liquid Canada balsam (75 g. of dry, pulverized balsam dissolved slowly in 100 cc. of xylene). After 2 or 3 min. drain off excess liquid. Place on filter paper, and dry 2 to 4 hr. The sections can be examined under fairly high magnification, and can be kept some time. Cracked and dust-covered sections may be repaired by dissolving in xylene and refreezing.—*Jean E. Conn.*

POLICARD, A. **La Methode de la Microincineration, Exposé Practique.** 6½ by 10 in., 50 pp. Paper. Herman et Cie., Paris. 11 francs.

The author of this well written and interesting booklet, by his many worthwhile studies on mineral distribution in cells and tissues, has in recent years, renewed interest in a field of investigation which had been dormant since the work of MacCallum at the beginning of the century. There is little doubt that micro-

incineration, if properly performed with tissues prepared by the frozen dehydration method, gives a reasonably true representation of mineral distribution in living cells and organisms. Furthermore, it is the only method extant which permits the appreciation of total mineral dispersion in biological materials. Altho the interpretation of such findings and their importance in physiology is difficult at present, they have given several new concepts of cellular organization in health and disease.

Professor Policard's monograph proceeds in an orderly and direct fashion from an historical resumé as introduction to the table of contents which takes the place of an index. The paragraphs dealing with fixation of tissues are perhaps too few, and possibly the frozen dehydration method could have been profitably included (instead of dismissing in a paragraph); but the author is not to be criticized severely in this instance since he is presumably writing primarily for readers not deeply concerned with intracellular localization of minerals.

Details of the technical procedures of incineration are dealt with carefully and thoughtfully. Many pitfalls in the technic are pointed out and artifacts and their causes described. Particular attention is given to the methods of examination of the preparations. The difficult question of interpretation of the incinerated tissue sections is handled cautiously and with no apparent evidence of wishful thinking.

Especially valuable is the third section of the booklet which is devoted to "Techniques complémentaires", for here the many accessory methods devised by workers in the field are described and discussed. The paragraphs devoted to chemical reactions, microphotometry and allied methods are well done in most respects. The bibliography is carefully selected and contains the titles of review articles on the subject.

Professor Policard's booklet should be of great value to the beginner who hopes to apply the method to biological problems. Perhaps one of its greatest uses is in bringing together the many laboratory artifices which have grown up around the basic technic.—*Gordon H. Scott.*

SCHENKEN, J. R., and McCORD, W. M. **A method for the examination of the cellular elements of body fluids. A collodion sac for concentration and paraffin-embedding.** *Amer. J. Clin. Path., Tech. Suppl.* **3,** 176–8. 1939.

Fill a 15 cc. chemically clean, dry centrifuge tube with fresh collodion, U.S.P. Pour out the collodion with a rotary motion so that the lip of the tube is uniformly and completely covered. Clamp the tube in an inverted position and drain 10 min. Rinse with water and separate the sac with the aid of a stream of water from a wash bottle. Test sac for leaks by filling it with water. Store it in 1:10,000 merthiolate. When ready for use, empty sac and fill it with fluid to be tested. Put a thin, moist glass rod in a 15 cc. centrifuge tube moistened with saline and use the rod to help in inserting the filled sac. Withdraw the glass rod. Fill the space around the sac with the fluid to be examined, or with saline. Fold the edge of the sac over the edge of the tube and secure it firmly by a cork, taking care to exclude air from the sac. Centrifuge. Remove sac and tie it off with No. 20 thread about 3 mm. above the sediment. Cut off the empty part of the sac and discard it. The tied portion may be fixed, dehydrated and embedded in paraffin by usual technics except that acetone and other collodion solvents cannot be used.—*George H. Chapman.*

SYLVÉN, B. **Experimentelle Beiträge zur Kenntnis der Färbung mit Mucikarmin nach P. Mayer.** *Zts. Wis. Mikr.,* **55,** 462–9. 1938.

The author studies first the effect of H-ion concentration on solutions of mucicarmine, or aluminum carminate using the following procedure: Prepare 1% solutions of mucicarmine, diluted with 10 parts of dist. water and a series of solutions of HCl ranging from pH 6.0 to pH 1.0. After 24 hr. the solutions with pH values above 3–4 appear clear and violet; those below appear red with a brownish flocculent ppt. The red color is due to acid carmine. Add a few drops of conc. NH_4OH to a 0.1% solution of mucicarmine. A thick violet red gelatinous ppt. forms, soluble in excess alkali. It consists of $Al(OH)_3$. Tap water dilutions from 1/400 to 1/60 produce the same result in 3–12 hr.; with dist. water, the dilutions last 6 days or more.

Next, the staining effect of mucicarmine solutions of different acidities is studied. Staining period 1 hr., pH 2–7; for acid carmine, 20 hr. The staining

capacity of mucicarmine lies between pH 4–7. A neutral solution produces diffuse red coloration, and at pH 2 the acid carmine stain takes place. Since chondroitin-H_2SO_4, as in cartilage, and mucoitin-H_2SO_4 are alkali soluble, lower acidities cannot be applied. Free radicals of these compounds may be bound to one or more of the trivalent Al-ions of Aluminum carminate.—*E. Barasch.*

WOLF, JAN. **Die Glyzerinmethode der Zelloidineinbettung.** *Zts. Wis. Mikr.*, **56,** 57–62. 1939.

This method was devised to shorten the time required for the celloidin technic. Reagents required: Glycerin (ordinary); Glycerin 31 Bé (100% glycerin is 30 Bé. *J. M. T.*); 8% celloidin in abs. alc. and ether. The fixed and washed material, (e.g. a piece of liver 3×6×6 mm.) is placed directly into ordinary glycerin for 6 hr.; from this it is transferred to anhyd. glycerin 31 Bé for an additional 6 hr. The bottle containing the glycerin and specimen should be turned upside down frequently to insure thoro infiltration.

The usual amount of 8% celloidin required for infiltrating and blocking the specimen is placed in a suitable container and the block of tissue, wiped free of excess glycerin, placed therein. Infiltration and inspissation of the celloidin is complete in 24 hr. The block can be mounted before transferring to 80% alcohol for hardening and preservation.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

BUGYI, B. **Reinheitsprüfung der histologischen Farbstoffe.** *Zts. Wis. Mikr.*, **55,** 198–210. 1938.

An easy method of testing for impurities in histological dyes by means of capillarity is described, and the results are presented for 38 different dyes. The only objectionable impurities are the diffusible substances which alter the color of the dye.

Cut strips of plain filter paper 2 cm. wide, 30 cm. long. Suspend 24 hr. in staining dish (10 cm. deep, 3–5 cm. wide) containing 15–20 cc. 1% solutions of the dye in dist. water, Ringer's solution, alcoholic, acid, and basic solutions.

Azocarmine seems to contain impurities. The impurity found with brilliant black is more diffusible than the dye (color, brown). Carmine was found pure, but acid carmine impure. Cresyl violet contains slight impurities. Dahlia is a compound dye producing a narrow red zone at the top of the diffusion picture. Ringer's and alc. solutions bring out impurities in erythrosin. A pink zone appears in a basic solution of methylene blue, due to the partially demethylated product. There is a violet impurity found in malachite green. Metanil yellow is not quite pure. Violet tinges are found with methyl green, probably due to parafuchsin derivatives. Nile blue sulfate is not a pure dye. Orange G seems impure; so does orcein D. Safranin may contain an impurity. There are red and blue zones in thionin. There is a red impurity in trypan blue.

Nearly all metachromatic dyes show impurities, but in general the purity of dyes has been greatly improved; dextrin, in fact, is almost never present.—*E. Barasch.*

FIGGE, F. H. J. **Melanin: a natural reversible oxidation-reduction system and indicator.** *Proc. Soc. Exp. Biol. & Med.*, **41,** 127. 1939.

Melanin was found by the author to be a natural redox system comparable to glutathione, riboflavine and cevitamic acid. The reduced form decolorized toluylene blue and other indicators; the oxidized form oxidized reduced indigo disulphonate, methylene blue and thionine. Oxidized melanin absorbed 70% of light (water given 0.0 absorption value); reduced with Na hydrosulfite only 25% was absorbed; reoxidized, 70% was again absorbed.—*M. S. Marshall.*

LEVINE, M. **Crown gall-like tumors induced with scharlach red on the plant, Kalanchoe.** *Proc. Soc. Exp. Biol. & Med.*, **40,** 599–603. 1939.

Crown-gall-like overgrowths, characterized by leafy shoots and roots, were produced on decapitated shoots of *Kalanchoe Daigremontiana* by scharlach red dissolved in ether. Other carcinogenic agents, as 1,2,5,6-dibenzanthracene, methylcholanthrene and benzopyrene, applied in lanolin caused injuries without inducing overgrowths. Stimulation of root formation was observed with some substances, including indole acetic acid and lanolin used alone.—*M. S. Marshall.*

STOW, W. P. **Rose bengal excretion and hippuric acid synthesis tests of liver function: a comparison.** *J. Lab. & Clin. Med.*, **24**, 866–8. 1939.

The author compares the results obtained with Delprat and Stowe's rose bengal excretion test and Quick's hippuric acid synthesis test in seven patients with liver dysfunction. Striking agreement is evident. It is proposed that the rose bengal test be used where speed is important, requiring only 1 hr. as compared to 12 hr. or more for the hippuric acid test. Quick's test is recommended where severe jaundice is present and in laboratories with limited reagents and apparatus. —*Parker R. Beamer.*

ANIMAL MICROTECHNIC

AURELL, G. **Kolophonium-Chininhydrochloridgemische als Einschlussmittel für sehr dicke Schnitte zu microskopischen Zwecken.** *Zts. Wis. Mikr.*, **55**, 256–73. 1938.

The purpose of this study is the introduction of Spalteholz' clearing method to micro-anatomy, supplementing present-day methods of reconstruction and replacing them in some cases.

The main requirement is a suitable embedding medium which is pure and does not crystallize and which has a refractive index similar to that of the tissues. To determine the refractive index of an organ, place the material in mixtures of different proportions of benzyl benzoate ($r = 1.568$) and methyl salicylate ($r = 1.536$). The combined index should fall somewhere between 1.560 and 1.550. Compare transparence of embedded sections by looking for cell walls. At the optimum refractive index they are invisible.

Proceed as follows: inject material for blood vessels, or stain with any dye except picric acid. Bleach 1 mm. sections of different organs with H_2O_2. Dehydrate completely in alcohol. Place sections in benzol. Transfer to clearing mixture, 12–24 hr. For microscopic study, embed material using anise oil ($r = 1.557$) as a solvent and colophonium with quinine-HCl as a medium. Dissolve quinine-HCl with gentle heating in anise oil containing measured amounts of colophonium. Let cool completely; add remainder of resin. Let stand for a few days. Place dry tissue into anise oil, then in medium. Leave it there one day. Section with freezing microtome. Study with stereoscopic microscope.—*E. Barasch.*

HOLMGREN, H. **Eine neue Methode zur Fixierung der Ehrlichschen Mastzellen. Mit besonderer Berücksichtigung der Chemie der Zellgranula.** *Zts. Wis. Mikr.*, **55**, 419–61. 1938.

Mast cells produce a secretion similar to heparin in its clotting effect and consisting of more or less water-soluble polyester sulfuric acid granules. This fact is the basis for the author's use of fixatives known to precipitate such compounds. Proceed as follows: Fix organs, such as liver, lung, kidney, spleen or ovary of various vertebrates, with a fresh basic 4% aq. solution of PbAc in a well closed bottle, 12–24 hr. Wash with dist. water. Transfer to 60% alcohol. Dehydrate, embed in paraffin. Between fixation and dehydration a second series may be washed for 24 hr., and a third series treated 24 hr. with a neutral solution of 10% formalin. To compare with the PbAc method, use for fixation: 1) quinine-HCl (4%) in conc. aq. solution and in 25, 12.5, and 5% alc. solution; 2) conc. aq. solution of $Ba(OH)_2$, and $BaCl_2$; 3) neutral solution of formalin (10%) followed by alcohol treatment or by washing for 24 hr. with water, or fixing in formol sublimate for 12 hr.; 4) Helly's solution; 5) Maximov's solution. Section blocks and stain, using toluidine blue, 1) in 1% aq. solution; 2) in 60% (1 g. in 99 cc.) alc. solution, which gives a specific metachromatic reaction with high molecular ester-H_2SO_4 compounds.

A heparin injection produces granules similar to those in Ehrlich's mast cells in endothelium which stain best with PbAc fixation. At a certain concentration of alc. solution (90%) of toluidine, high molecular carbohydrates with few ester groups no longer stain, whereas the polyester granules retain their staining property.—*E. Barasch.*

LANDAU, E. **Contribution aux methodes myeliniques.** *Bull. d'Histol. Appl.* **15**, 181–5. 1938.

The author has refined his earlier technic for staining myelin sheaths so that it gives good results for small pieces as well as hemispheres and whole brains. The procedure is as follows: (1) Fix 10–14 days in 10% formol. (2) Rinse 24 hr. in

running water. (3) Fix 24 hr. at 25–30° C. in the following solution: $K_2Cr_2O_7$, 5 g.; 10% aq. $FeCl_3$ or iron alum, 20 drops; dist. water, 100 cc. (4) Wash in running water 6–12 hr., according to the size of the piece. (5) Dehydrate successively. (6) Abs. alc. (7) Chloroform, change 2 or 3 times. (8) Embed in paraffin. (9) Section at 15μ. (10) Attach the sections with gelatin or dist. water. (11) Remove the paraffin and mordant 3–6 hr. at 25–30° C. in a solution of 10% $FeCl_3$ or 10% iron alum. (12) Rinse 2 sec. in dist. water. (13) Stain several hours in 1% aq. hematoxylin at 25–30° C. (14) Wash 1–2 hr. in running water. (15) Check the differentiation in K ferrocyanide from time to time under the microscope (K ferrocyanide 2.5 g. and sat. aq. soln. Li_2CO_3, 100 cc.). (16) Wash several hours in running water. (17) Counterstain, if desired, with Hollburn's "Kernechtrot" (Kernechtrot, 0.1 g.; $Al_2(SO_4)_3$, 5.0 g.; dist. water, 100 cc.) 5 min. (Domagk's mixture). (18) Dehydrate, mount in balsam.—*Jean E. Conn.*

LILLIE, R. D. **Experiments on the solubility of hemosiderin in acids and other reagents during and after various fixations.** *Amer. J. Path.*, **15**, 225–39. 1939.

The acid solubility of iron-containing pigment in sectioned tissues from man and laboratory animals was studied after treatment with a number of commonly used fixatives. The state of the Fe in hemosiderin was shown to be ferric. Fixation in acid fixatives which contained no formalin either removed hemosiderin or extracted the iron from it, leaving a non-ferrous brown pigment. After fixation in formalin or in formalin-containing fixatives, hemosiderin became more resistant to subsequent extraction by acids. In general, the efficacy of various acids used in aq. solutions to extract hemosiderin after formalin were, in order of decreasing efficacy: oxalic, sulfuric, nitric, formic, and hydrochloric. There was considerable variability in the time required for removal of the pigment. Four hours in 10% H_2SO_4 (aq.) at 25° C. was sufficient in many preparations but some required either higher temp. (37°) or a longer treatment. The age of the pigment had little influence on its speed of solution, but that which had coarse granules resisted solution longer. The iron-free pigment, hemofuchsin, was sometimes present after acid-containing fixatives or after treatment of fixed tissue with warm acids. This pigment was believed to be derived from hemosiderin by loss of iron. The evidence indicated that the iron existed originally in combination with protein.—*H. A. Davenport.*

LADEWIG, P. **Über eine einfache und vielseitige Bindegewebsfärbung. (Modifikation der Mallory-Heidenhainschen Methode).** *Zts. Wis. Mikr.*, **55**, 215–7. 1938.

A simple method for staining connective tissue elements evenly and constantly without precipitation is described by the author. The procedure is as follows: fix material, preferably in formalin. Make paraffin sections 5–10 μ thick. Place in xylol, and run thru decreasing concentrations of alcohol to water. Place in Weigert's iron hematoxylin, 3–5 min. Wash briefly with dist. water. Transfer sections to 5% phosphotungstic acid, 1–3 min. This stains the connective tissue elements light red, the nuclei remaining dark brown. Wash briefly with dist. water. Place for 4 min. in staining fluid (1 g. acid fuchsin, added to 100 cc. of Mallory's original solution, boiled briefly and filtered when cold). Wash briefly with dist. water. Transfer directly to 96% alcohol, 1 min., moving sections slightly. Place in abs. alcohol, xylol, and balsam.

Results: nuclei, dark brown; connective tissue, from blue to carmine; parenchyma, gray blue or brown; nervous tissue, the same; muscle, reddish brown; erythrocytes, light orange; fibrin, bright red; mucus and secretions, blue; amyloid, glassy light blue; colloid, violet to red; calcium, red; keratin, red; eleidin, orange; transitional tissue, violet; keratohyalin, dark brown.—*E. Barasch.*

LADEWIG, P., and DESSAU, F. **Ergebnisse mit einer neuen Bindegewebsfärbung.** *Zts. Wis. Mikr.*, **55**, 211–4. 1938.

The authors have investigated Ladewig's modification of Mallory's connective tissue stain using the "azan" procedure for control sections. Collagenous fiber tracts, in vascular walls, in scar tissue, in inflammatory changes, and in some tumors, stain red, violet or blue, the same shades appearing in similar sections which all indicate some fibrinoid proliferation in a broad sense. Fuchsinophil connective tissue indicates injury to the tissue, but some injuries are not thus represented. The distinctness with which fibrin and paraplastic substances stain

makes the procedure useful for the diagnosis of necrosis and growth processes. Since erythrocytes stain yellow to orange, i.e., differently from all other elements, the slightest hemorrhages can thus be detected. Amorphous calcium and bone trabeculae appear bright red, while osteoid tissue is bluish. Changes in ovary and uterus which take place mostly in the connective tissue elements may be easily followed. Parenchymatous organs and epithelial structures are not stained intensively enough to obscure the finer details of cell structure. Main and parietal cells in the gastric fundus are easily distinguished, as are also the three types of cellular components of the hypophysis, and keratin, eleidin, and keratohyalin.—*E. Barasch.*

MOLLIER, G. **Eine Vierfachfärbung zur Darstellung glatter und quergestreifter Muskulatur und ihrer Beziehung zum Bindegewebe.** *Zts. Wis. Mikr.*, **55**, 472–3. 1938.

Since azocarmin does not stain myofibrils and nuclear structure in sufficient detail, the author proposes the following modification of the "azan" staining method: Transfer the sections from 10% alcohol to orcein (12 hr.). Wash well with dist. water until no more color comes off, and place in Weigert's hematoxylin for 1–3 min. Wash with dist. water. Differentiate briefly using 0.1% HCl in 70% alcohol until nuclei are distinct. Wash 15 min. in running tap water. Stain 15–30 min. with cold azocarmin G. Wash with dist. water. Place 3–6 hr. in 5% phosphotungstic acid, changed 2–3 times until collagenous fibers are completely decolorized. Wash with dist. water. Stain 15–30 min. with naphthol green B (1 g. in 100 cc. dist. water, 1 cc. glacial acetic acid). Transfer directly to 96% alcohol for 30 sec., stirring to prevent precipitation of green. Pass thru alcohol, xylol, balsam. Result: chromatin, nucleoli, deep blue; cytoplasm, bluish-red; myofibrils, deep red with sharp contours; elastic fibers, dark brown; collagenous fibers, olive; erythrocytes, bright red. Start with Weigert's stain, if elastic stain is to be omitted. This stain is prepared as follows: Solution I: dissolve 1 g. hematoxylin in 100 cc. 96% alcohol, add 0.2 g. $NaIO_3$ and 62 cc. dist. water. Solution II: 4 cc. liquid ferrous bichlorate, 96 cc. dist. water, 1 cc. officinal HCl. Immediately before use 1.5 parts of solution I and 1 part of solution II are poured together and mixed. Do not use mixture longer than 2 hr. —*E. Barasch.*

MONNE, L. **Über Vitalfärbung tierischer Zellen mit Rhodaminen.** *Zts. Wis. Mikr.*, **55**, 143–9. 1938.

The author presents the results of his vital staining experiments with electrically neutral rhodamine B, Schultze (1923) No. 573. When injected into amebae, the ground cytoplasm is stained diffusely. In other cells, e.g., spermatocytes and spermatids of *Helix*, oocytes of *Helix* and *Arachnida*, frog muscles and *Opalina ranarum*, the dye stains the ground cytoplasm diffusely, the mitochondria more intensely.

Between crossed Nicol prisms of the polariscope cells stained with rhodamine B and 6G (Schultz No. 571)—which stain alike—show an orange-red fluorescence. Oocytes and embryos of the *Ascidia, Styela partita*, contain differently staining primary tissues: blastomeres forming ectoderm and muscles do not stain; those giving rise to the intestine stain intensively; and those forming the notochord, weakly. The circumnuclear plasma of the entomeres does not stain.—*E. Barasch.*

PRICKETT, C. O. and STEVENS, CORNELIA. **The polarized light method for the study of myelin degeneration as compared with the Marchi and Sudan III methods.** *Amer. J. Path.*, **15**, 241–50. 1939.

The lumbosacral and sciatic nerve trunks from 142 rats were studied as follows: 71 were used as controls, the remaining 71 had the right nerves cut and allowed to degenerate for periods of 24, 48, 72, 120 and 216 hr. respectively in different groups of animals. For study by polarized light and Sudan III, the tissues were fixed in neutral formalin (Merck); for Marchi, in Müller's fluid. Frozen sections were used for polarized light and Sudan III. The polarized light method was found to be rapid and accurate; the certainty of recognizing changes characteristic of degeneration was present 24 hr. after transection. The earliest degeneration shown by the Marchi method was 72 hr., and by Sudan III, 120 hr. after transection. The Marchi method was inconsistent as compared with results obtained by

polarized light. The Sudan III method was consistent but failed to show the earlier changes. The observations of Setterfield, Sutton, and Baird (Stain Techn. **11**, 41. 1936), relative to the superiority of polarized light over Marchi's staining in the detection of degenerating myelin sheaths of peripheral nerves, were confirmed.—*H. A. Davenport.*

ROSKIN, G. **Sur la diagnostic cytologique différentiel des cellules cancéreuses.** *Bull. d'Histol. Appl.* **15**, 20–3. 1938.

Staining with the leucobase of methylene blue (Rongalitweiss of Unna) leaves cells from malignant tissues colorless, but normal tissues are stained an intense blue. This was tested on tumors of the white mouse, rat, chicken, guinea pig, and man.

To prepare the "rongalitweiss", use a 5% solution of methylene blue (to which 7 drops of 25% HCl have been added). To 10 cc. of this solution add 0.3 g. of rongalite (of which the quality is important). Heat carefully until colorless or pale yellow. The pH should not be lower than 2.41. Use tissues dried in the air. Stain 2 min. Wash quickly but carefully in O-free water (kept free of O by a thin coat of liquid paraffin). Dry on filter paper and seal with paraffin.

Normal tissue can even be distinguished macroscopically from malignant tissue by the difference in color. This method is suggested as a supplement to present methods of diagnosis.—*Jean E. Conn.*

TURNER, OSCAR A. **A manual of neurohistologic technique.** *J. Lab. & Clin. Med.*, **24**, 735–48. 1939.

This is the first of several installments dealing with general considerations, stains for nerve cells, myelin sheaths, neurofibrils, fats, glia, connective tissue, pituitary and pineal body, spirochetes and miscellaneous methods. One chapter near the end of the article is to deal with formulas and the entire article is to be supplemented with a list of selected references.

In this installment fixation of tissues, removal of tissue, laboratory equipment and gelatin embedding are discussed. Procedures for the following staining methods are given:

(1) For staining myelin sheaths: Pal-Weigert, Spielmeyer, Weil rapid method, Wolter-Kulschitzky, and the Marchi method for degenerating myelin.

(2) For staining neurofibrils: Bielschowsky's silver method and Cajal's method.

Certain advantages and disadvantages of each method are also mentioned together with hints as to the technics of performing them.—*Parker R. Beamer.*

TURNER, OSCAR A. **A manual for neurohistologic technique.** *J. Lab. & Clin. Med.*, **24**, 871–87. 1939. (Second installment in series)

Complete procedures and comments are given regarding the following stains:

(1) For fats. Sudan III, Herxheimer's method (utilizing a derivative of Sudan III) and Nile blue sulfate.

(2) For staining interstitial cells, a division into metallic and non-metallic stains has been made for convenience. *Non-metallic methods*: Mallory's phosphotungstic-acid-hematoxylin stain, Bailey's ethyl-violet-orange-G stain for fibrillary neuroglia, Holzer's glia-fiber stain, and Alzheimer-Mann's method for neuroglia. *Metallic methods*: Cajal's gold sublimate method and Corten's modification, Kanzler's modification of Hortega's method for microglia, Penfield's second modification of Hortega's silver carbonate method, Wilder's method for glia and nerve fibers and Fincher's silver carbonate method.

Glia stains on paraffin sections are mentioned briefly with the comment that they usually are not very reliable and rarely are they selective.—*Parker R. Beamer.*

WEISSCHEDEL, E. and JUNG, R. **Die anatomische Auswertung und das Studium der secundären Faserdegeneration nach lokalisierter subcorticaler Ausschaltung durch Elektrokoagulation.** *Zts. Anat. u. Entwgesch.*, **109**, 374–95. 1939.

The staining of degenerating myelin is discussed in the light of carefully controlled experiments with the tract degeneration in the central nervous systems of cats and rabbits. The original Marchi method is compared with the chlorate-osmic-formalin method (Swank and Davenport, Stain Techn., **10**, 87–90. 1935.) The latter method is considered to have advantages over the original Marchi by

requiring less osmic acid, by saving time, and by giving a clearer background. It was found to be less suited for beginners because of lack of uniform impregnation. Counterstaining with cresyl violet from Bender and Holbein after bleaching sections with $KMnO_3$ and oxalic acid was best. Authors call for differentiation of cresyl violet in 1% alc. HCl. (This is apparently an erroneous quotation from Swank and Davenport and should be 0.01%.) They recommend alum-cochineal for counterstaining after the regular Marchi stain. A rapid embedding method is given which employs 1 part anhyd. $CuSO_4$ in 4 parts acetone for dehydration; followed by 3 to 5 changes of abs. alc., at 2 to 3 min. intervals with shaking; alcohol-ether 1 to 2 min.; 2 to 3% celloidin, two changes of 5 min. and 10 min. respectively, during which time the 4 to 6 mm. slices of tissue must be turned carefully and the fluid kept agitated. The individual slices of the original piece of tissue are then built up on a previously prepared celloidin base 3 to 5 mm. thick into the composite block, the whole immersed in thick celloidin and hardened in chloroform. The solvent action of alcohol-ether and celloidin solutions on degenerating myelin is thereby avoided.—*H. A. Davenport.*

ZACH, O. **Die naturgemässe Zelloidin-Einbettung.** *Zts. Wis. Mikr.*, **55**, 299–307. 1938.

The author describes the disadvantages of the common celloidin embedding method and proposes the following process for improvement of irregular hardening and tearing: Soak the material well in celloidin, as usual. Place it in an embedding vessel containing 8% celloidin solution. Spread well. For inspissation place material into desiccator containing a spoonful of $CaCl_2$. No lid is needed. Open desiccator every 12 hr. Remove contents, aerate well, replace contents. The $CaCl_2$ also prevents much evaporation of alcohol. In 2–3 days the celloidin sets in a soft gel covered by a tougher skin. Free it from the edges with a small knife. When only traces of ether remain, take out the $CaCl_2$ every 12 hr. and burn it. Replace the dry salt. In a few days the celloidin may reach the desired hardness. Free it from embedding vessel. If bottom is still soft, wait 1–2 days more. For some kinds of tissue, the block may have to be dried further. Place blocks in 90% alcohol containing a little safranin, 24 hr. Pour off the alcohol. Place in an alcohol-turpineol mixture, 3:1, 24 hr., alcohol-turpineol, 1:1, 24 hr., turpineol, several days. Add a little safranin to mixtures, preventing decolorization. Change turpineol until alcohol from blocks no longer reddens it. The blocks may be sectioned a few days later with a dry knife, cutting at a 45° angle.

It is easy to cut 14 μ sections. With care, they may be made 10 μ thick; 6 μ sections are more difficult, but even 4 μ sections may be made of brain and gland tissue.—*E. Barasch.*

PLANT MICHROTECHNIC

KOONZ, C. H. and RAMSBOTTOM, J. M. **A method for studying the histological structure of frozen fruits. 1. Poultry.** *Food Res.*, **4**, 117–28. 1939.

A Pyrex desiccator containing P_2O_5 serves as the drying chamber. The parts are sealed with vaseline diluted with kerosene. It is connected thru two dry ice traps to a Cenco-Hyvac pump operating with a light oil. Samples of the frozen poultry are cut several millimeters in diameter and about 1 cm. long. After 24 hr. the desiccator stopcock is closed, the pump stopped and the desiccator allowed to warm up to room temp. The dried and fixed tissues are taken out and immediately plunged into melted paraffin. (If taken out while extremely cold they will absorb considerable moisture from the air.) If properly dehydrated the tissues will sink to the bottom of the paraffin within a few minutes. Sections should not be floated on water because this tends to produce coarse breaks. Sections are preferably placed on dry slides.—*George H. Chapman.*

MICROÖRGANISMS

GIEMSA, G. **Einfache Dauerfärbung von Hautpilzen und Mikroorganismen mit Azureosinglycerin. Bemerkungen zur Arbeit von Erich Hoffmann, diese Wschr. 1938, 1622.** *Klin. Woch.*, **18**, 133–4. 1939.

The Giemsa stains give quite different results in tissue staining depending on whether or not they are used in fluids in which they dissociate. The author

observed and reported this phenomenon many years before E. Hoffmann was puzzled by the apparent reversal in staining reaction.—*V. W. Kavanagh.*

HEWITT, REDGINAL. **A staining technic for demonstrating avian malaria parasites in tissue sections.** *Amer. J. Hyg.* **29,** *Section C,* 115–7. 1939.

A modification of the Wollbach's Giemsa stain is given. After fixation in Zenker formol and imbedding in paraffin, sections of liver, spleen and bone marrow are cut 5–10μ thick. The sections are mordanted for 30–60 min. in 2.5% $K_2Cr_2O_7$, then stained 24 hr. in the following solution: dist. water, 100 cc.; 0.5% $NaHCO_3$, 2–4 drops; methyl alcohol, 3 cc.; Giemsa's stain (Azure I eosin, National Aniline, 3 g.; Azure II, 0.8 g.; methyl alcohol, 250 cc.; glycerol 200 cc.) 2.5 cc. Differentiate in 70% alcohol and dehydrate in xylol-acetone mixtures. Mount in neutral balsam or cedar oil. This technic gives good results in staining tissues of canaries infected with *Plasmodia cathemarium.* Mature erythrocytes stain orange and the parasites within them stain blue. The chromatin of the parasite does not stain red as in blood films.—*John T. Myers.*

KUROTCHKIN, T. J. **The value of Gram's stain for differentiation of pathogenic fungi.** *Chinese Med. J.,* Suppl. **2,** 337–41. 1938.

One hundred twenty species of all known groups of pathogenic fungi were stained by Gram's method, employing 0.02% aq. safranin for counterstain. Four groups were differentiated by staining: (1) Fungi completely Gram-negative, e.g. *Trichosporon*; (2) Fungi showing Gram-negative mycelia and Gram-positive spores, e.g. *Penicillium, Aspergillus*; (3) Fungi completely Gram-positive, e.g. *Trichophyton,* and *Microsporon*; (4) Fungi partially Gram-positive, e.g. *Sporotrichum.* References are given.—*Sara A. Scudder.*

MORTON, H. E. **The survival of microorganisms in fixed and stained preparations.** *Amer. J. Clin. Path., Tech. Suppl.,* **3,** 68–70. 1939.

Among about 40 preparations tested by flaming three times and then testing for viability in infusion broth, *Staphylococcus aureus, S. albus,* Pneumococcus Type I, *Streptococcus faecalis* and *Mycobacterium phlei* survived. Two cultures survived 1 min. in basic fuchsin; one in Hucker's gentian violet 1 min.; four in aqueous safranin 1 min.; and two in Loeffler's alkaline methylene blue 15 min. These results indicate that care should be taken in handling such preparations.—*George H. Chapman.*

OGDEN, M. A. **A new method of making smears for hematologic, cytologic and bacteriologic examinations.** *Amer. J. Clin. Path., Tech. Suppl.,* **3,** 183. 1939.

Put one drop of test fluid near the edge of a slide. Hold at an angle of 35° and smear on a second slide from the center to the end. Place 3–5 drops on the other half of the slide, and spread, making a thick smear. This provides a thick and a thin smear on the same slide. If cells are numerous the thin half can be examined. If cells are scarce the thick half will be more suitable.—*George H. Chapman.*

v. HORVATH, J. **Eine neue Silbermethode für die Darstellung der erregungsleitenden Elemente der Ciliaten.** *Zts. Wis. Mikr.,* **55,** 113–22. 1938.

The purpose of this method is the representation of the nervous structures of ciliates, independent of sun or artificial light exposure, using the reduction of formalin by NaOH. The procedure is as follows: fix 2–60 sec. with concentrated formalin, (diluted 3–4 times with culture fluid—i.e. approximately 10% formalin). Centrifuge and pour off solution without washing. Place 3–4 min. in 1–2% or 10% aq. solution $AgNO_3$. Centrifuge, pour off $AgNO_3$ solution without washing. Place in 1–3% aq. solution NaOH. A brown or black ppt. is formed; that previously formed by formalin is dissolved. Centrifuge material briefly at high speed. Wash in series of alcohols, then in alc. gylcerol; if alcoholic material turns gray under cover glass, omit alcohol and use dilute gylcerol.

This method is not specific for nervous elements as it also stains the supporting network of ciliates. It may be applied to the study of higher organisms.—*E. Barasch.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the July number of this Journal.

STAINS CERTIFIED JUNE 1, 1939 TO AUG. 31, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Janus green B	NJ–7	58%	As vital blood stain	June 5, 1939
Erythrosine	NEr–5	88%	As histological counterstain	June 5, 1939
Nile Blue A	LNb–2	76%	As stain for fats and fatty acids	June 6, 1939
Malachite green	CMg–4	98%	As histological and bacteriological stain	June 19, 1939
Wright Stain	CWr–12	....	As blood stain	June 23, 1939
Malachite green	LMg–4	96%	As histological and bacteriological stain	June 26, 1939
Methylene blue	LA–8	85%	As histological and bacteriological stain and as constituent of blood stains	June 26, 1939
Pyronin G	NP–7	55%	As constituent of Pappenheim's stain	June 26, 1939
Sudan III	NY–6	90%	As fat stain	July 18, 1939
Hematoxylin	FH–15	....	As histological and cytological stain	July 18, 1939
Aniline blue WS	NK–4	....	As histological stain	Aug. 7, 1939
Brilliant cresyl blue	NV–16	60%	For vital staining of blood	Aug. 7, 1939
Eosin Y	NE–14	87%	As histological stain and as constituent of blood stains	Aug. 16, 1939
Crystal violet	NC–20	89%	As histological, cytological and bacteriological stain, and in bacteriological media	Aug. 22, 1939
Malachite green	NMg–7	94%	As histological and bacteriological stain	Aug. 23, 1939

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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